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# Reaction of Mixed Valence State Cytochrome Oxidase with Oxygen in Plant Mitochondria

## A STUDY BY LOW TEMPERATURE FLASH PHOTOLYSIS AND RAPID WAVELENGTH SCANNING OPTICAL SPECTROMETRY

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### ABSTRACT

**The reaction of mixed valence state cytochrome oxidase ($Cu_A^{2+}a^{3+}\cdot Cu_B^{+}a_3^{2+}$) with $O_2$ at 173 K has been investigated in purified potato mitochondria by low temperature flash photolysis and rad wavelength scanning optical spectrometry in the visible region. The kinetics of the reaction have been analyzed simultaneously at six wavelength pairs (586-630, 590-630, 594-630, 604-630, 607-630, and 610-630 nanometers) by nonlinear optimization techniques, and found to proceed by a two-species sequential mechanism. The "pure" difference spectra of the two species, $I_M$ and $II_M$, relative to unliganded mixed valence state cytochrome oxidase have been obtained. The difference spectrum of species $I_M$ is characterized by a peak at 591 nanometers, with a shoulder at 584 nanometers and a trough at 602 nanometers, and that of species $II_M$ by an α band split into a prominent peak at 607 nanometers and a small side peak at 594 nanometers. Evidence is presented to suggest that these two bands arise from $O_2^- \rightarrow Cu_B^{2+}$ and $O_2^- \rightarrow a_3^{2+}$ charge transfer transitions which would imply that $O_2^-$ forms a bridging ligand between $Cu_B$ and the iron atom of cytochrome $a_3$ in species $II_M$. The kinetics of the reaction and the spectral characteristics of species $I_M$ and $II_M$ obtained with the potato mitochondrial system are compared and contrasted with data in the literature on the beef heart mitochondrial system.**

Cytochrome oxidase (EC 1.9.3.1) catalyzes the terminal reaction, the four equivalent reductions of molecular $O_2$ to water, in the respiratory electron transport chain of all higher organisms. The minimum functioning unit of Cyt oxidase is thought to consist of two A type haems, Cyt *a* and $a_3$, differing only in the nature of their axial ligands, and two copper atoms, $Cu_A$ and $Cu_B$ (34).

Much interest has centered on the reactions of both fully reduced ($Cu_A^{+}\,a^{2+}\cdot Cu_B^{+}\,a_3^{2+}$) and mixed valence state ($Cu_A^{2+}\,a^{3+}\cdot Cu_B^{+}\,a_3^{2+}$) Cyt oxidase with $O_2$ at sub-zero temperatures (4, 5, 7–10, 12–17, 22–25). Kinetic studies of the reaction of mixed valence state Cyt oxidase with $O_2$ in beef heart mitochondria (7, 8) or in the isolated form (10) revealed two intermediates of A and C type noted compound $A_2$ and compound C or $C_2$ (7, 8, 10). Numerical analysis of the kinetics followed optically at low temperature according to the method of Chance *et al.* (3, 4) with a multichannel spectrometer (6) suggested the existence of a third intermediate from a three step sequential mechanism (14)

$$E_M + O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} I_M \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} II_M \xrightarrow{k_{+3}} III_m \qquad (1)$$

(where $E_M$ is the unliganded mixed valence state Cyt oxidase).[3]

Recently, Denis (22) and Clore (12) resolved in time and wavelength two C-type species in beef heart mitochondria, $C_{606}$ ($II_M$) and $C_{610}$ ($III_M$) and discussed their involvement in the above model.

In the present paper we have investigated the reaction of mixed valence state Cyt oxidase with $O_2$ in purified potato mitochondria by low temperature flash photolysis and rapid scan spectrometry (11, 20, 26) in order to compare and contrast the spectral and kinetic properties of the potato and beef heart mitochondrial systems.

## MATERIALS AND METHODS

**Biochemical Methods.** Potato mitochondria, freshly prepared by the method of Ducet *et al.* (28), were suspended at 293 K in a medium containing 20 mM sodium phosphate buffer (pH 7.4) and 7 mM succinate. Mitochondria were purified by centrifugation on a discontinuous density gradient of saccharose and left for 10 min (*i.e.* until all the $O_2$ in the preparation was exhausted). The preparation was then cooled down to 273 K and saturated with CO. Ethylene glycol was added (final concentration 30% v/v) and the preparation resaturated with CO in order to ensure full anaerobiosis and CO saturation. The preparation was then stored in an air-tight syringe at 253 K until further use when it was transferred into 2 mm optical path length cuvettes previously deaerated with CO for optical studies.

The mixed valence state Cyt oxidase-CO complex in which Cyt *a* and $Cu_A$ are in the ferric and cupric states respectively, and Cyt $a_3$ and $Cu_B$ in the ferrous and cuprous states, respectively, was prepared by adding potassium ferricyanide (final concentration 5 mM) at 253 K in the dark by the addition of $O_2$ saturated 30% v/v ethylene glycol (containing 2 mM $O_2$ when saturated at 253 K (3, 10). The sample was then transferred to a cooling system consisting of an ethanol bath temperature regulating system (Neslab Instruments) at 193 K, and the suspension stirred vigorously in the dark until the viscosity increased and freezing occurred. This procedure prevents ligand exchange between $O_2$ and the CO-inhibited system (4, 7, 8). The cuvette was then transferred to the Dewar flask of the spectrometer through which thermoregulated $N_2$ (Air Liquide, Philips) of the desired temperature flowed. A copper constantan thermocouple was used for temperature mea-

[3] Species $I_M$ is also known as compound $A_2$ and species $II_M$ and $III_M$ both belong to the class C group of compounds (see Refs. 7–10 and 12–17).

surements.

**Flash Photolysis.** The reaction of unliganded mixed valence state Cyt oxidase with $O_2$ was initiated by flash photolysis of the mixed valence state Cyt oxidase-CO complex at 173 K by a 500 J xenon flash lamp (Cunow) with a pulse width of 1 ms. The flash was approximately 99% saturating. CO did not recombine to any detectable extent in the presence of the relatively high $O_2$ concentration employed, as shown by control experiments where repeated flashes over the course of the reaction with $O_2$ only produced about 1% further photolysis of the CO complex, the intermediates formed in the reaction with $O_2$ not being susceptible to photolysis at the flash intensity used. The temperature of 173 K was chosen so as to allow a direct comparison with the kinetic data obtained for the mixed valence state Cyt oxidase-$O_2$ reaction in intact beef heart mitochondria (12, 14).

**Rapid Wavelength Scanning Spectrometry.** To collect absorbance changes over a wide spectral range with good time resolution, we used a home built CD66 rapid wavelength scanning spectrometer. The optical design and performance characteristics of the CD66 rapid scan spectrometer have already been described in detail (11, 20, 26) so that only a schematic diagram of the optical system external to the CD66 monochromator, the signal-processing system and the low temperature attachment are shown here (Fig. 1).

The CD66 spectrometer was used in a single beam mode. Each stored spectrum was obtained averaging 400 successive transient spectra. Each transient spectrum was defined by 256 points in the wavelength interval 484 to 631.4 nm (*i.e.* a spectral resolution of 0.576 nm per point). The recording time for each point was set at 7 $\mu$s and the time required to record a single transient spectrum was set at 3 ms. A delay time of 7 ms between the recording of each transient spectrum was used. Thus the scanning rate was 100 transient spectra per s. The averaged spectrum was transferred in 0.30 s from the memory of the Histomat S data acquisition system (Intertechnique) to magnetic tape for further analysis.

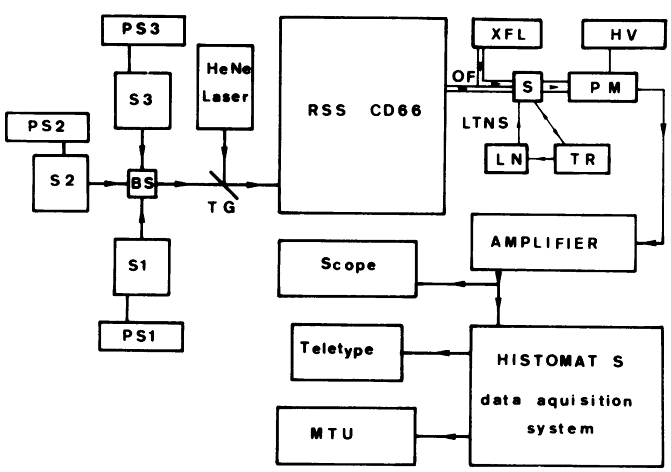


FIG. 1. Schematic diagram of the optical system external to the CD66 rapid scanning monochromator, the signal-processing system, and the low temperature attachment. The spectral lamp S1 (Philips HgZnCd) is used in regulating and controlling the rapid wavelength scanning spectrometer. The tungsten-halogen lamp S2 and the xenon-arc lamp S3 (Osram XBO 150) are used as light sources for absorption measurements. PS1, PS2, and PS3 are the power supplies of S1, S2, and S3, respectively. The monochromatic light beam from the Helium-Neon laser (Spectraphysics) is superimposed on the measuring beam, and provides the synchronization signal for the recorded spectra. BS is a beam splitter; TG, a transparent quartz plate; RSS, the rapid scanning CD66 monochromator; OF, optical fibers; XFL, xenon flash lamp for photolysis; TR, temperature-regulating device (Air Liquide, Philips); LTNS, low temperature nitrogen stream; S, sample; PM, photomultiplier (EMI 9558 BQ); HV, stabilized high voltage power supply of the photomultiplier; MTU, magnetic tape unit.

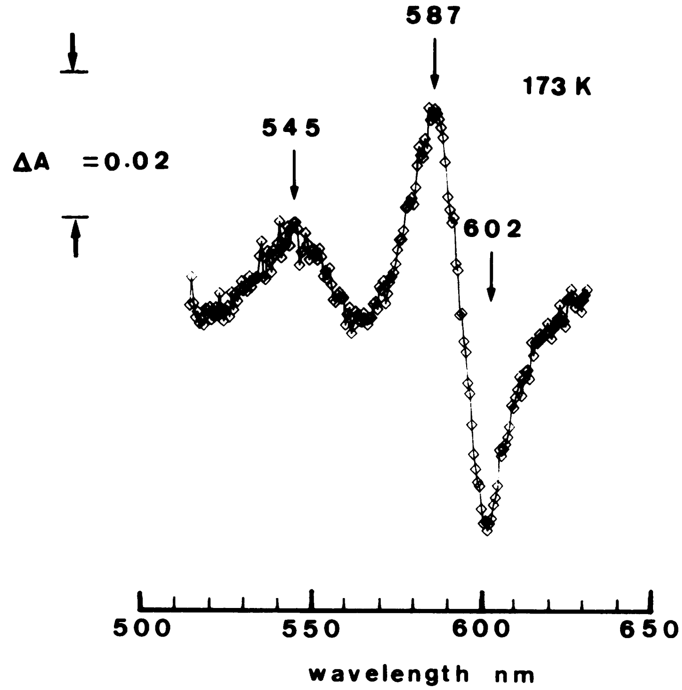


FIG. 2. CO compound difference spectrum at 173 K. It corresponds to $a_3^{2+}$ CO $Cu_B^+$ minus $a_3^{2+}$ $Cu_B^+$ and results from the difference between the spectra recorded, respectively, before and after the flash. Each memorized spectrum was the average of 400 successive spectra swept at the rate of 100 spectra per s and digitized in 7 $\mu$s per point, with 256 points between 484 and 631.4 nm. CO was about 98% photodissociated with a single flash from a 500 J xenon flash lamp. Reaction sample: purified potato mitochondria 13 mg protein/ml containing 5 $\mu$M Cyt oxidase (calculated from $\Delta\epsilon^{605}_{red\text{-}ox} = 17$ mM$^{-1}$ cm$^{-1}$ [29]), ethylene glycol 30% v/v, 1 mM $O_2$, 0.6 mM CO, 10 mM $K_3Fe(CN)_6$, 1.8 mM succinate, 9 mM Pi.

After CO flash photolysis up to 900 averaged spectra were stored on magnetic tape according to the procedure outlined above.

**Data Analysis.** Only 200 points per spectrum (513.5 to 631.4 nm) were kept for the treatment of the data on magnetic tape and their analysis in order to facilitate data handling. Difference spectra were obtained relative to the first spectrum stored after CO flash photolysis which corresponded to unliganded mixed valence state cytochrome oxidase. All difference spectra were computed using 630 nm as the reference wavelength (*i.e.* a digital dual wavelength technique was employed).

Six wavelength pairs (586–630, 590–630, 594–630, 604–630, 607–630 and 610–630 nm) were selected for kinetic analysis. The methods of kinetic analysis, numerical integration and nonlinear optimization were as described previously (13, 14).

For the treatment of the data and the display of both spectra and kinetic curves we used respectively a CII 10 070 computer and a Benson 122 plotter at the Centre de Calcul du Pharo de l'Université d'Aix-Marseille II. Kinetic analysis were carried out at the University College London Computer Centre.

## RESULTS

**Time Resolved Optical Difference Spectra.** Figure 2 shows the CO compound difference spectrum ($a_3^{2+}$ CO $Cu_B^+$ minus $a_3^{2+}$ $Cu_B^+$) corresponding to the difference between the spectra recorded, respectively, before and after the flash. The extent of the peak (587 nm) to trough (602 nm) *A* deviation indicates the good quality of the preparation of the oxygenated sample (minimum CO bound loss) and of the photodissociation itself.

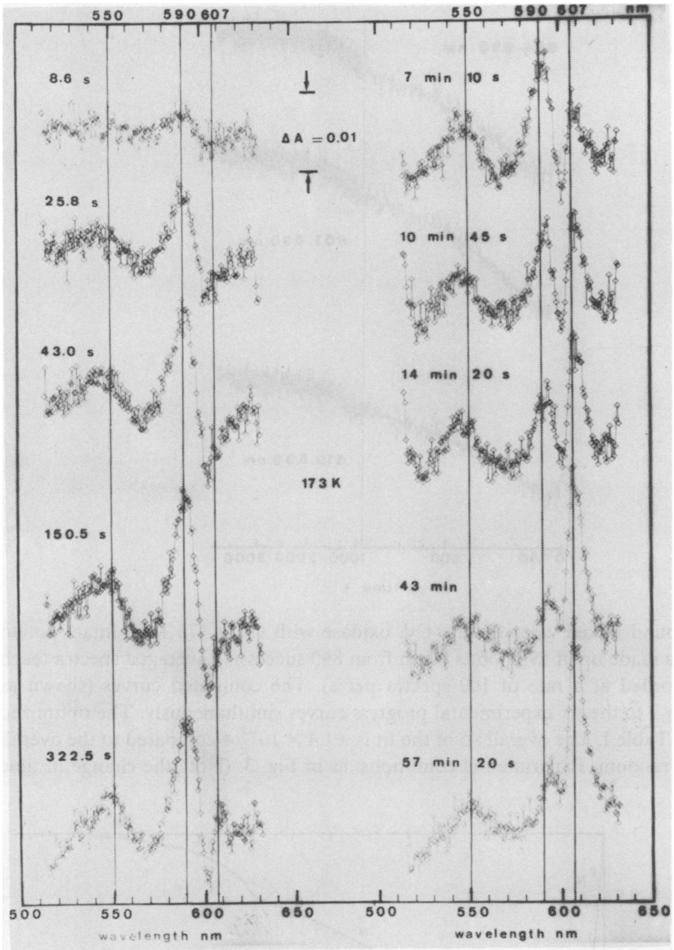


FIG. 3. Optical difference spectra (reaction sample minus unliganded mixed valence state Cyt oxidase) in the visible region obtained at successive times in the reaction of membrane-bound mixed valence state Cyt oxidase with $O_2$ at 173 K in intact potato mitochondria. Each spectrum is obtained by averaging 400 successive spectra recorded at a rate of 100 spectra per s. The experimental conditions are: potato mitochondria, 13 mg/ml containing 5 $\mu$M Cyt oxidase (calculated from $\Delta\epsilon^{605}_{\mathrm{redox}} = 17$ mM$^{-1}$ cm$^{-1}$ [29]), 3.5 mM succinate, 10 mM sodium phosphate buffer (pH 7.4), 30% (v/v) ethylene glycol, 5 mM $K_3Fe$ CN)$_6$, 0.6 mM CO, and 1 mM $O_2$.

Typical time resolved difference spectra (reaction sample minus unliganded mixed valence state Cyt oxidase, species $E_M$) in the visible region illustrating the progress of the reaction of mixed valence state Cyt oxidase with $O_2$ at 173 K in purified potato mitochondria are shown in Figure 3. Two distinct optical species are resolved in time and wavelength. The spectra up to 150 s illustrate the formation of species $I_M$ which is characterized by a peak at 591 nm with a shoulder at 584 nm and a trough at 601 in the $\alpha$ region and a peak at 547 nm in the $\beta$ region. The spectra taken thereafter illustrate the conversion of species $I_M$ into species $II_M$[3] which is characterized by a split $\alpha$ band with a large peak at 607 nm and a side peak at 594 nm, and a 550 nm peak in the $\beta$ region. This second species behaves as a real end point for the reaction.

**Kinetic Analysis.** The progress curves at 586–630, 590–630, 594–630, 604–630, 607–630, and 610–630 nm illustrating the kinetics of the mixed valence state Cyt oxidase-$O_2$ reaction at 173 K are shown in Figure 4, together with the best fit computed curves (continuous line) for a two species model stated as:

$$E_M + O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I_M \xrightarrow{k_{+2}} II_M \tag{2}$$

where $E_M$ is unliganded mixed valence state Cyt oxidase. This model was fitted simultaneously to the six experimental progress curves using the equation:

$$\Delta A_i(t) = \sum_l \alpha_i(l)\cdot F_l(t)\cdot S_i \tag{3}$$

where $\Delta A_i(t)$ is the absorbance change at the ith wavelength pair at time $t$, $F_l(t)$, the concentration of the lth species at time $t$ obtained by numerical integration of the differential equations derived for the kinetic scheme given by Equation 2; $S_i$, a scale factor; and $\alpha_i$ (l) the relative extinction coefficient of the lth species at the ith wavelength pair defined by the equation:

$$\alpha_i(l) = \frac{\Delta\epsilon_i\,(l - z)}{\Delta\epsilon_i\,(x - z)} \tag{4}$$

where $\Delta\epsilon_i$ $(l - z)$ and $\Delta\epsilon_i$ $(x - z)$ are the molar difference extinction coefficients between species l and $z$, and species $x$ and $z$, respectively. (Thus, $\alpha_i(x) = 1.0$ and $\alpha_i(z) = 0$.) For the 586–630, 590–630, and 594–630 nm to traces species $x$ and $z$ correspond to species $I_M$ and $E_M$, respectively; for the 604–630, 607–630, and 610–630 nm traces species $x$ and $z$ correspond to species $II_M$ and $E_M$, respectively. The parameters optimized during the fitting procedure were the rate constants $k_{+1}$, $k_{-1}$, and $k_{+2}$, the relative extinction coefficients of species $II_M$ at 586–630, 590–630 and 594–630 nm, the relative extinction coefficients of species $I_M$ at 604–630, 607–630 and 610–630 nm, and the scale factors for each wavelength pair (*i.e.* a total of 15 optimized parameters for six experimental progress curves).

The overall SD of the fit for the model given by Equation 2 is $\pm 1.4 \times 10^{-3}$ $A$ compared to the overall standard error of the data of $\pm 2\times 10^{-3}$ $A$, the distribution of residuals is random and the optimized parameters are well determined. The values of the optimized parameters together with their $\mathrm{SD_{ln}}$[4] and confidence limits are given in Table I. The computed time course of species $E_M$, $I_M$, and $II_M$ is shown in Figure 5.

**Pure Difference Spectra of Species $I_M$ and $II_M$**—In Figure 6 the computed "pure" (*i.e* in 100% concentration) difference spectra of species $I_M$ and $II_M$ minus unliganded mixed valence state Cyt oxidase (species $E_M$) are shown. These difference spectra are obtained by the solution of a set of linear simultaneous equations of the form

$$\Delta A_i(t) = \sum_l F_l(t)\cdot\Delta\epsilon_i(l\text{-}E_M) \tag{5}$$

for each wavelength $i$, where $\Delta A_i(t)$ is the observed difference in absorbance at the ith wavelength between the reaction sample at time t and the reference spectrum of unliganded mixed valence state Cyt oxidase; $F_l(t)$ the computed concentration for the lth species determined by numerical integration of the differential equations derived for the kinetic scheme given by Equation 2, using the optimized values of the rate constants given in Table 1; and $\Delta\epsilon_i$ (l-$E_M$) the molar difference extinction coefficient at the ith wavelength between the lth species and unliganded mixed valence state Cyt oxidase (species $E_M$) obtained by the solution of Equation 5. The spectral characteristics in the visible region of the difference spectra of species $I_M$, species $II_M$, and the mixed valence state Cyt oxidase-CO complex minus unliganded mixed valence state Cyt oxidase are collected in Table II and compared to those of the corresponding species obtained with membrane-bound and soluble Cyt oxidase from beef heart mitochondria. The extent of absorbance changes in spectra of species $I_M$ and $II_M$ (in 100%

[4] Abbreviations: $\mathrm{SD_{ln}}$, standard deviation of the natural logarithm of an optimized parameter; EPR, electron paramagnetic resonance; l.w.h.h., line width at half-height.

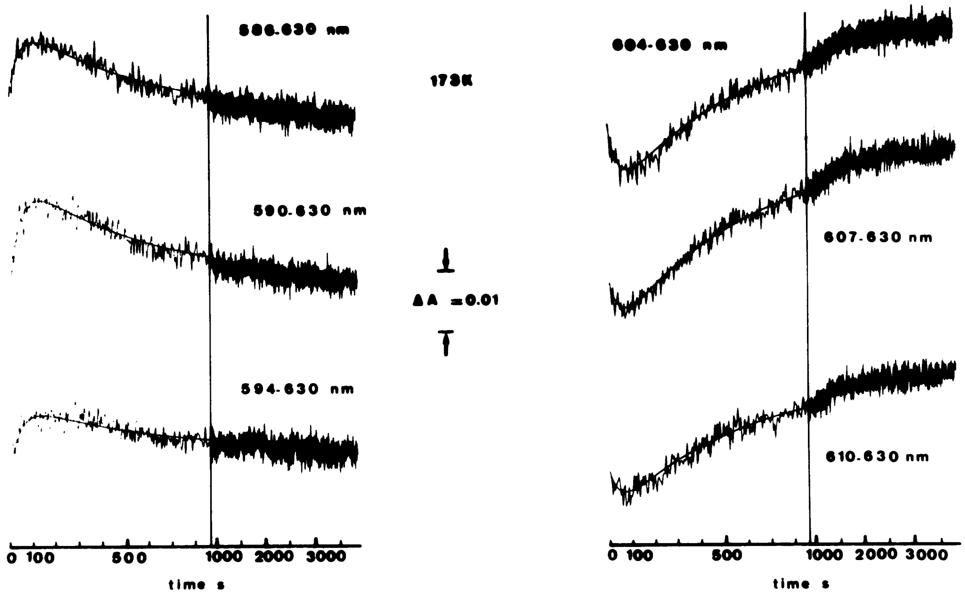


FIG. 4. Observed and computed kinetics of the reaction of membrane-bound mixed valence state Cyt oxidase with $O_2$ at 173 K in intact potato mitochondria as measured at six wavelength pairs. Each experimental curve is made up of 890 points taken from 890 successive averaged spectra (each averaged spectrum being obtained by averaging 400 successive spectra recorded at a rate of 100 spectra per s). The computed curves (shown as continuous lines) are obtained by fitting the kinetic scheme given by equation 2 to the six experimental progress curves simultaneously. The optimized values of the parameters used in calculating the computed curves are given in Table I. The overall SD of the fit is $\pm 1.4 \times 10^{-3}$ $A$ compared to the overall standard error of the data of $2 \times 10^{-3}$ $A$, and the distribution of residuals is random. Experimental conditions: as in Fig. 3. (Note the change in time scale at 870 s).

Table I. *Optimized Values of the Rate Constants ($k_i$), Relative Extinction Coefficients ($\alpha_i[I]$) and Scale Factors ($S_i$) Together with Their $SD_{ln}$ and Confidence Limits Obtained by Fitting the Kinetic Scheme Given by Equation 2 Simultaneously to the Experimental Progress Curves at Six Wavelength Pairs in Fig. 4*

For the 586–630, 590–630, and 594–630 nm wavelength pairs, $\alpha_i$ (1) = $\Delta\epsilon_i$ ($1$-$E_M$)/$\Delta$ $\epsilon_i$ ($I_M$-$E_M$) so that $\alpha_i$ ($I_M$) = 1.0 and $\alpha_i$ ($E_M$) = 0. For the 604–630 and 610–630 nm wavelength pairs, $\alpha_i$ (1) = $\Delta$ $\epsilon_i$ ($1$-$E_M$)/$\Delta\epsilon_i$ ($II_M$-$E_M$) so that $\alpha_i$ ($II_M$) = 1.0 and $\alpha_i$ ($E_M$) = 0.

| Parameter | Dimensions | Value | $SD_{ln}$ | Confidence Limits | |
|---|---|---|---|---|---|
| | | | | 5% | 95% |
| $k_{+1}$ | $M^{-1}$ $s^{-1}$ | 16.1 | 0.305 | 9.77 | 26.6 |
| $k_{-1}$ | $s^{-1}$ | 0.00596 | 0.699 | 0.00188 | 0.0188 |
| $k_{+2}$ | $s^{-1}$ | 0.00244 | 0.316 | 0.00145 | 0.00408 |
| $\alpha_{586}(II_M)$ | | −0.135 | 0.282 | −0.0849 | −0.215 |
| $\alpha_{590}(II_M)$ | | 0.0649 | 0.344 | 0.0368 | 0.114 |
| $\alpha_{594}(II_M)$ | | 0.191 | 0.303 | 0.116 | 0.314 |
| $\alpha_{604}(I_M)$ | | −1.27 | 0.132 | −1.02 | −1.58 |
| $\alpha_{607}(I_M)$ | | −0.614 | 0.200 | −0.442 | −0.853 |
| $\alpha_{610}(I_M)$ | | −0.603 | 0.203 | −0.432 | −0.842 |
| $S_{586}$ | $mM^{-1}$ | 3.60 | 0.249 | 2.39 | 5.42 |
| $S_{590}$ | $mM^{-1}$ | 5.11 | 0.285 | 3.20 | 8.16 |
| $S_{594}$ | $mM^{-1}$ | 2.86 | 0.284 | 1.79 | 4.57 |
| $S_{604}$ | $mM^{-1}$ | 2.81 | 0.170 | 2.12 | 3.72 |
| $S_{607}$ | $mM^{-1}$ | 4.04 | 0.122 | 3.31 | 4.94 |
| $S_{610}$ | $mM^{-1}$ | 2.97 | 0.121 | 2.43 | 3.62 |

concentration) referred to the unliganded mixed valence state $E_M$ have to be compared to those of the CO compound difference spectrum in Figure 2.

## DISCUSSION

Rapid scan spectrometry has been developed in our laboratory to study highly scattering biological materials like mitochondria, producing small absorbance changes, less than 0.1 $A$ (20, 26, 27).

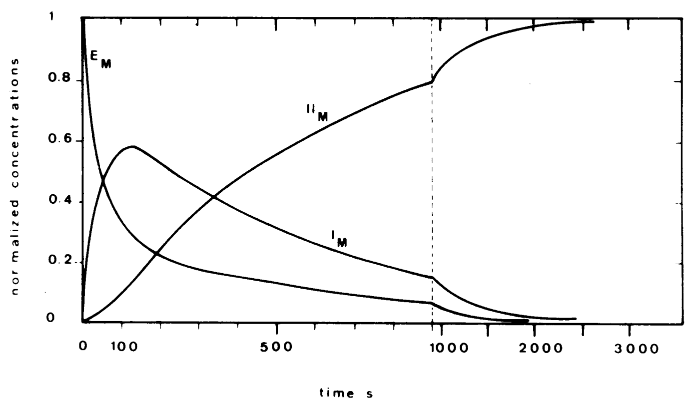


FIG. 5. Computed time courses of unliganded mixed valence state Cyt oxidase (species $E_M$) and species $I_M$ and $II_M$ in the reaction of membrane-bound valence state Cyt oxidase with $O_2$ at 173 K in intact potato mitochondria. The computed time courses of the species are obtained by numerical integration of the differential equations derived for the kinetic scheme given by Equation 2 using the optimized values of the rate constants given in Table I. The initial conditions are: [$E_M$] = 5 $\mu$M, [$O_2$] = 1 mM.

To our knowledge, other applications in the biological field are related only to reactions in solution at room temperature and giving rise usually to far larger absorbance changes (18, 19, 30, 32, 35, 36).

Essentially two ways of referring absorbance changes have previously been used to study the low temperature reaction of Cyt oxidase with $O_2$. The first one was to compare the transient states to the CO-bound Cyt oxidase either fully reduced or in the mixed valence state. It has been associated with the multichannel dual wavelength technique developed by Chance *et al.* (3, 4, 6–8) which is a purely kinetic technique allowing one to record up to 8 wavelength pairs simultaneously (3, 4, 15). The second one, introduced by Denis (22, 24) consists in taking as reference spectrum

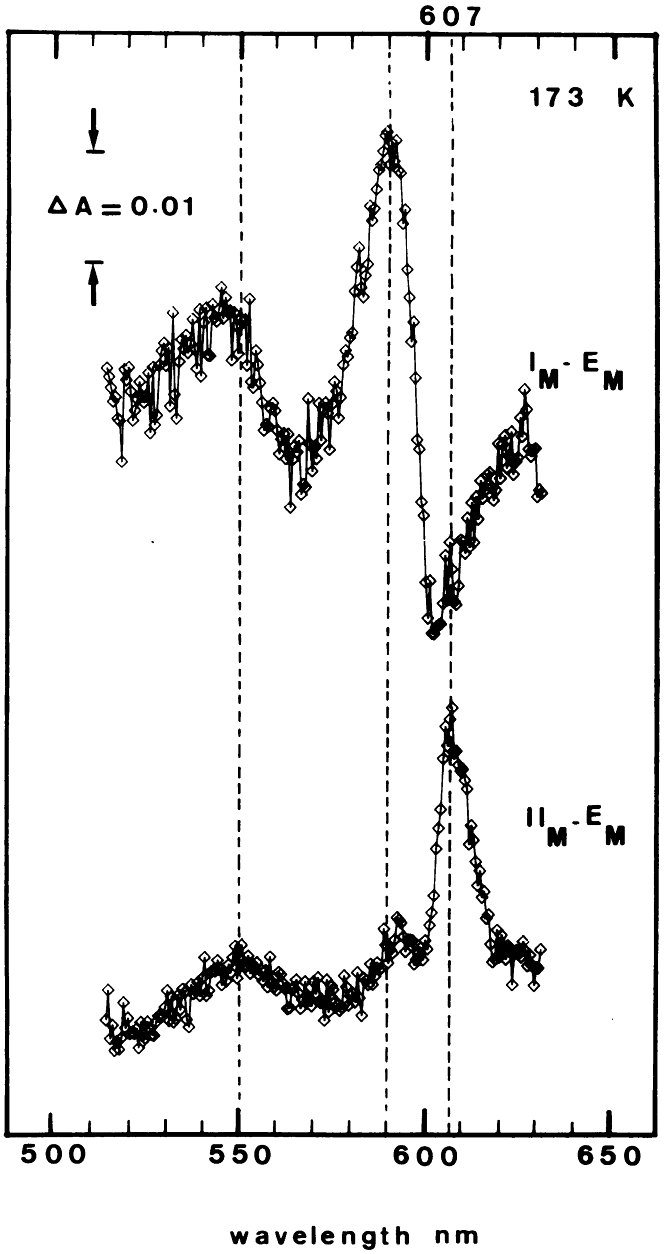


FIG. 6. Computed pure difference spectra of species $I_M$ and $II_M$ minus unliganded membrane bound mixed valence state Cyt oxidase in intact potato mitochondria. The pure difference spectra of the species (*i.e.* in 100% concentration) are calculated from the difference spectra in Fig. 3 by using Equation 5 as described in the text. The concentration of each species is 5 $\mu$M.

the unliganded state memorized after 100% CO dissociation at about 153 K. The reaction with $O_2$ is then initiated by raising the temperature to the range 173 to 193 K. This reference provided transient difference spectra easier to interpret and allowed the resolution of two C-type components (24). A temperature effect of this technique has been described in detail by Denis and Clore (25). Recently, Clore *et al.* (16, 17) used a modification of this second method to correlate optical and EPR spectra by memorizing the unliganded state spectrum at 77 K and checking optically the evolution of the reaction after successive warming and cooling cycles between 77 and 173 or 193 K. The repeated cooling/warming technique suffers from (a) poor time resolution (a minimum 10–20 s interval between the recording of successive spectra) and (b) the possible introduction of spectral distortions inherent in the technique due to changes in the crystallization state of the sample occurring on each warming and cooling cycle.

The rapid scan spectrometry technique employed here combines the requirements for both kinetic studies (single flash at constant temperature and good time resolution, ~4 s) and spectral characterization of intermediates (wide spectral range scanned with a good resolution, ~0.6 nm per point, and absorbance changes referred to the unliganded state). Furthermore, such a complete set of data with reasonable signal to noise ratios (Figs. 3 and 4) belongs to a single experiment.

A comparison of the kinetic and spectral features of the reaction of mixed valence state Cyt oxidase with $O_2$ in the potato and beef heart mitochondrial systems reveals a number of noteworthy features:

1. Only two species are seen in the reaction in the intact potato mitochondrial system, in contrast with the beef mitochondrial system where two C-type species ($C_{606}$ and $C_{610}$) have been resolved (22).

2. The reaction is much slower in the potato mitochondrial system than in the beef heart mitochondrial system at 173 K, the rate constants in the former (Table I) being smaller than the corresponding rate constants in the latter (12, 14) by a factor ranging from 3 to 6.

3. There are small but significant differences between the potato and beef heart mitochondrial systems in the positions of the peaks and troughs of the difference spectra of species $I_M$ and the mixed valence state Cyt oxidase-CO complex minus unliganded mixed valence state Cyt oxidase (see Table II).

4. A comparison of the peak to trough intensities in the $\alpha$ region of the pure difference spectra of species $I_M$ and the mixed valence state Cyt oxidase-CO complex minus unliganded mixed valence state Cyt oxidase shows that in the potato mitochondrial system the peak to trough intensities of the species $I_M$ and mixed valence state Cyt oxidase-CO complex difference spectra are approximately equal (see Figs. 2 and 6), whereas in the beef heart mitochondrial system, the peak to trough intensity of the species $I_M$ difference spectrum is approximately double that of the mixed valence state Cyt oxidase-CO complex difference spectrum (Fig. 3 of Ref. 12).

5. The difference spectrum of species $I_M$ minus unliganded mixed valence state Cyt oxidase looks more complex in the potato mitochondrial system than in the mammalian one due to the presence of a shoulder at 584 nm on the low wavelength side of the 591 nm band (Fig. 3).

6. The difference spectrum of species $I_M$ minus unliganded mixed valence state Cyt oxidase exhibits a split $\alpha$ band in the potato mitochondrial system with a prominent peak at 607 nm and a side peak at 594 nm (Figs. 3 and 6 and Table II). For this reason, we will refer to this species as $II_M$ ($C_{607}^{594}$) in the discussion which follows. In contrast, in the beef heart mitochondrial system the difference spectrum of species $C_{606}$ ($II_M$) exhibits only a single peak in the $\alpha$ region at 606 nm (Table II and Refs. 12, 22).

These optical particularities of the intermediates compounds in potato mitochondria confirms the previous findings of Denis and Bonner (23).

As discussed on previous occasions (5, 7–10), the spectral features of species $I_M$ can be entirely accounted for by the formation of an end-on bond between the iron atom of Cyt $a_3$ and $O_2$ in which there is delocalization of electrons not only from the iron atom of Cyt $a_3$ but also from $Cu_B$ to $O_2$. This situation could be represented by the configuration $Cu_B^{1+\delta_1}\, a_3^{2+\delta_2}\, O_2^{-(\delta_1+\delta_2)}$, where $(\delta_1 + \delta_2) \sim 1$, $\delta_1 < 0.5$, and $\delta_2 > 0.5$. (It should be noted that the maximum separation between the iron atom of Cyt $a_3$ and $Cu_B$ is of the order of 5 Å on account of the strong anti-ferromagnetic coupling between Cyt $a_3$ and $Cu_B$ with an exchange coupling constant of $-J \geqslant 200$ cm$^{-1}$ [1]).

The most unusual and in many ways the most interesting of the

Table II. *Characteristics of the Difference Spectra of Species $I_M$, Species $II_M$, and the Mixed Valence State Cyt Oxidase-CO Complex Minus Unliganded Mixed Valence State Cyt Oxidase (Species $E_M$)*

Observations were made in the visible region with membrane-bound Cyt oxidase in intact potato and beef heart mitochondria, and soluble Cyt oxidase from beef heart mitochondria. Abbreviations: p, peak; t, trough; s, shoulder

| Species | Membrane-bound Cyt Oxidase | | | | Soluble Cyt Oxidase from Beef Heart Mitochondria | |
|---|---|---|---|---|---|---|
| | Potato mitochondria | | Beef heart mitochondria | | | |
| | $\beta$ | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ | $\alpha$ |
| Mixed valence state | | | 550 | 588 612[a] | | |
| Cyt oxidase-CO | | | 545 | 587 607[b] | | |
| complex | 545 p | 587 p 602 t | 547 p | 586 p 605 t[c] | 547 p | 586 p 610 t |
| | | 584 s | 546 | 590 606[b] | | |
| $I_M$ | 547 p | 591 p 602 t | 549 p | 590 p 608 t[d] | 549 p | 592 p 614 t |
| | | | 549 | 606[b] | | |
| $II_M$ | 550 p | 594 p 607 p | 550 p | 606 p[d] | | 605 p[e] |
| | | | | 609 p[a] | | 609 p[cf] |

[a] Ref. 9.
[b] Ref. 22.
[c] Ref. 10.
[d] Ref. 12.
[e] Ref. 17.
[f] M. Denis, unpublished result.

species formed in the reactions of Cyt oxidase with $O_2$ are those belonging to the class C group of compounds (which includes species $C_{606}$ ($II_M$) and $C_{610}$ ($III_M$). In Cyt oxidase derived from beef heart mitochondria the class C group of compounds is characterized by an intense absorption band in the 605 to 610 nm region (extinction coefficient ~15-20 $mM^{-1}$ $cm^{-1}$ [3, 15]) and a narrow bandwidth (line width at half-height = 350-550 $cm^{-1}$ [12, 17]) characteristic of a charge transfer band. The identity of species $II_M$ has been discussed at great length on the basis of EPR (17) and optical data (5, 7-10, 17, 22) like the 655 nm band (2, 21, 23, 24) and all the available evidence to date point to the configuration $Cu_A^{2+}\ a^{3+} \cdot Cu_B^{2+}\ a_3^{2+} \cdot O_2^-$. In the beef heart mitochondrial system where only a single $\alpha$ band is seen, the intense absorption band centered around 606 nm for species $II_M$ ($C_{606}$) was attributed to a charge transfer band arising from interaction between Cyt $a_3^{2+}$, $Cu_B^{2+}$, and $O_2^-$ (5, 17). The presence in the potato mitochondrial system of a split $\alpha$ band with peaks at 594 and 607 nm in species $II_M$ ($C^{594}_{607}$) indicates the presence of two electronic events. This suggests that the 594 and 607 nm bands of species $II_M$ ($C^{594}_{607}$) arise from $O_2^- \rightarrow Cu_B^{2+}$ and $O_2^- \rightarrow a_3^{2+}$ charge transfer transitions differing in energy by ~4.3 kJ $mol^{-1}$. Such an assignment is consistent with the known $O_2^{2-} \rightarrow Cu^{2+}$ charge transfer band at 584 nm in oxyhaemocyanin (33) and the $O_2^- \rightarrow a_3^{2+}$ charge transfer band around 590 nm in species $I_M$ (17). In species $II_M$ obtained with Cyt oxidase derived from beef heart mitochondria, the energies of the $O_2^- \rightarrow Cu_B^{2+}$ and $O_2^- \rightarrow a_3^{2+}$ charge transfer transitions would be approximately equal because only a single absorption band is seen. This is further supported by the observation that the bandwidth of the 605 to 606 nm band in species $II_M$ (l.w.h.h. = 350-500 $cm^{-1}$, Refs. 12 and 17) obtained with Cyt oxidase derived from beef heart mitochondria is approximately the same as that of the 594 nm band (l.w.h.h. = 280 $cm^{-1}$) of species $II_M$ ($C^{594}_{607}$).

The presence of both $O_2^- \rightarrow Cu_B^{2+}$ and $O_2^- \rightarrow a_3^{2+}$ charge transfer bands in species $II_M$ ($C^{594}_{607}$) implies that $O_2^-$ forms a bridging ligand between $Cu_B^{2+}$ and Cyt $a_3^{2+}$ with a configuration, taking into account electron delocalization within the $Cu_Ba_3 \cdot O_2$ unit, best represented by

$$Cu_B^{1+\delta_1} \text{——} O^{-} \overset{(\delta_1+\delta_2)}{\text{————}} O \text{——} a_3^{2+\delta_2}$$

where $(\delta_1 + \delta_2) \sim 1$, $\delta_1 > 0.5$, and $\delta_2 < 0.5$.

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